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Title CHARACTERISATION OF GENE FUNCTION USING DOUBLE STRANDED RNA INHIBITION

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2.	Patent application number *(The Patent Office will fill in this part)*	9827152.1
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	Patents ADP number *(if you know it)*	7USU911002
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4.	Title of the invention	CHARACTERISATION OF GENE FUNCTION USING DOUBLE STRANDED RNA INHIBITION
5.	Name of your agent *(if you have one)* "Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)* Patents ADP number *(if you know it)*	BOULT WADE TENNANT 27 FURNIVAL STREET LONDON EC4A 1PQ 42001

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CHARACTERISATION OF GENE FUNCTION USING DOUBLE STRANDED RNA INHIBITION

The present invention is concerned with characterization or identification of gene function using double stranded RNA inhibition (dsRNAi) and methods of identifying DNA responsible for inducing a specific phenotype in a cell and a method of assigning function to known gene sequences.

It has recently been described in Nature Vol 391, pp.806-811, February 98, that introducing double stranded RNA into a cell results in potent and specific interference with expression of endogenous genes in the cell and which interference is substantially more effective than providing either RNA strand individually as proposed in antisense technology. This specific reduction of the activity of the gene was also found to occur in the nematode worm Caenorhabditis elegans (*C. elegans*) when the RNA was introduced into the genome or body cavity of the worm.

The present inventors have utilized this technique and applied it further to devise novel and inventive methods of assigning functions to genes or DNA fragments, which have been sequenced in various projects, such as, for example, the human genome project and which have yet to be accorded a particular function and for use in identifying DNA responsible for conferring a particular phenotype.

Therefore, according to a first aspect of the present invention there is provided a method of identifying DNA responsible for conferring a phenotype in a cell which method comprises a) constructing a cDNA or genomic library of the DNA of said cell in a suitable vector between two promoters capable of

promoting transcription of said cDNA or DNA to double stranded (ds) RNA upon binding of an appropriate transcription factor to said promoters, b) introducing said library into one or more of said cells comprising said transcription factor, and c) identifying and isolating a desired phenotype of said cell comprising said library and identifying the DNA or cDNA fragment from said library responsible for conferring said phenotype. Preferably said cell is derived from or contained in an organism.

In a preferred embodiment of the invention the library may be organised into hierarchical pools prior to step b) such as to include, for example, gene families.

According to a further aspect of the invention there is also provided a method of assigning function to a known DNA sequence which method comprises a)identifying a homologue(s) of said DNA in a cell, b) isolating the relevant DNA homologue(s) or a fragment thereof from said cell, c) cloning said homologue or fragment into an appropriate vector between two promoters capable of promoting transcription of dsRNA upon binding of an appropriate transcription factor to said promoters, d) introducing said vector into said cell from step a) comprising said transcription factor, and e) identifying the phenotype of said cell compared to wild type.

The cell according to the invention may be derived from or contained in an organism. Where the cell is contained within an organism, the organism may be adapted to express the appropriate transcription factor. The organism may be any of a plant, animal, fungus or yeast but preferably may be the nematode worm *C. elegans*, which may be any of a wild type, a nuc-1 or pha-ts mutant of *C. elegans* or a combination

of said mutations. In an alternative embodiment the DNA or cDNA library or the DNA homologue or fragment thereof may, advantageously, be transfected or transformed into a microorganism, such as a bacterial or yeast cell, which may be fed to the organism, which is preferably the nematode worm *C. elegans*. In this embodiment of the invention the microorganism may be adapted to express the appropriate transcription factor. Preferably, the microorganism is *E. coli*.

In each aspect of the invention, preferably said library or said DNA homologue or DNA fragment is constructed in a suitable DNA vector which comprises a sequence of nucleotides which encode said transcription factor. Alternatively, said transcription factor is encoded by a further vector. Alternatively, the cell or organism may have been mutated to express said transcription factor. Preferably, the vector used in the method according to the invention comprises a selectable marker. Preferably, the selectable marker comprises a nucleotide sequence encoding sup-35 or a fragment thereof which sequence is located between two promoters on said vector capable of expressing dsRNA upon binding of an appropriate transcription factor to said promoters, and which marker is selectable when contained in a pha-1 mutant *C. elegans*. Preferably, the promoters are T7 promoters.

Compounds can, advantageously, in said method be added to said cell or organism for the purposes of screening for desired phenotypes, such as for example, resistance or sensitivity to the compound when compared to wild type. The promoters are preferably inducible. The transcription factor may in some embodiments be phage derived, such as for example, a T7 polymerase driven by a phage promoter. However,

when *C. elegans* is utilised a worm specific promoter can be used. Preferably, the *E. coli* strain is an RNAseIII and even more preferably an RNase negative strain.

The present invention also includes a method of validating clones identified in yeast two hybrid vector experiments which experiments are well known to those skilled in the art and which experiments were first proposed by Chien *et al.* (1991) to detect protein - protein interactions. The method according to the invention comprises providing a construct including the DNA encoding a protein identified in a two hybrid vector experiment, which construct is such that said DNA is provided between two promoters capable of promoting transcription of said DNA to double stranded RNA upon binding of an appropriate transcription factor to said promoters, transforming a cell, such as a bacterial cell or alternatively transforming an organism comprising said transcription factor with said construct and identifying a phenotypic change in said cell or organism, which may be *C. elegans* or the like, compared to wild type. Preferably, the transcription factor is inducible in the cell or organism. Preferably, the promoter is a phage polymerase promoter and said transcription factor is a RNA polymerase, and preferably T7 polymerases. Also encompassed with the scope of the present invention are vectors used to transform said cells or organisms and the cells or organisms themselves.

In a further aspect of the present invention there is provided a method of alleviating pest infestation of plants, which method comprises a) identifying a DNA sequence from said pest which is critical either for its survival, growth,

proliferation or reproduction, b) cloning said sequence from step a) or a fragment thereof in a suitable vector between two promoters capable of transcribing said sequence to RNA or dsRNA upon binding of an appropriate transcription factor to said promoters, and c) introducing said vector into the plant.

Thus, advantageously, the method according to the invention provides a particularly selective mechanism for alleviating pest infestation, and in some cases parasitic infestation of plants, such that when the pest feeds on the plant it will digest the expressed dsRNA in the plant thus inhibiting the expression of the DNA which is critical for its growth, survival, proliferation or reproduction. In a preferred embodiment, the pest may be any of Tylenchulus ssp. Radopholus ssp., Rhadinaphelenchus ssp., Heterodera ssp., Rotylenchulus ssp., Pratylenchus ssp., Belonolaimus ssp., Canjanus ssp., Meloidogyne ssp., Globodera ssp., Nacobbus ssp., Ditylenchus ssp., Aphelenchoides ssp., Hirschmenniella ssp., Anguina ssp., Hoplolaimus ssp., Heliotylenchus ssp., Criconemellassp., Xiphinemassp., Longidorus ssp., Trichodorus ssp., Paratrichodorus ssp., Aphelenchs ssp. The DNA sequence or fragment thereof according to this aspect of the invention may be cloned between two tissue specific promoters, such as two root specific promoters.

A further aspect of the invention concerns the vector used in each of the methods of the invention for constructing said library, which vector comprises two identical promoters oriented such that they are capable of initiating transcription of DNA sequence located between said promoters to dsRNA upon binding of an appropriate transcription factor to said

promoters. Preferably, the expression vector comprises a nucleotide sequence encoding a selectable marker. In one embodiment the nucleotide sequence encoding said selectable marker is located between two identical promoters oriented such that they are capable of initiating transcription of DNA located between said promoters to double stranded RNA upon binding of an appropriate transcription factor to said promoters. Preferably, the selectable marker comprises a nucleotide sequence encoding sup-35, for introduction into *C. elegans* having a pha-1 mutation.

Preferably, the transcription factor comprises either a phage polymerase which binds to its corresponding promoter or a *C. elegans* specific promoter and even more preferably T7 polymerase. Preferably, the vector includes a multiple cloning site between said identical promoters.

In a further aspect of the invention there is provided an expression vector for expressing an appropriate transcription factor for use in a method according to the invention which vector comprises a sequence of nucleotides encoding said transcription factor operably linked to suitable expression control sequences. Preferably, the expression control sequences include promoters which are inducible, constitutive, general or tissue specific promoters, or combinations thereof. Preferably, the transcription factor comprises a phage, polymerase, and preferably T7 RNA polymerase.

A further aspect of the invention provides a selection system for identifying transformation of a cell or organism with a vector according to claim 38 which system comprises a vector according to claim 38 wherein said selectable marker comprises a nucleotide sequence capable of inhibiting or preventing

expression of a gene in said cell or organism which gene is responsible for conferring a known phenotype preferably said nucleotide sequence is a part of or identical to said gene conferring said known phenotype, and which nucleotide sequence is itself located between two identical promoters capable of initiating transcription of double stranded RNA upon binding of an appropriate transcription factor thereto. Alternatively, the nucleotide sequence comprises a nucleotide sequence which is a part of or identical to said gene sequence which confers a known phenotype on said cell or organism, and which is such that following integration of said vector by homologous recombination in the chromosome of said cell or organism said sequence inhibits expression of said gene sequence conferring said known phenotype. Preferably, according to this embodiment the nucleotide sequence comprises stop codons sufficient to prevent translation of the nucleotide sequence following integration into said chromosome. Preferably, the known gene sequence comprises a sup-35 gene or a fragment thereof which is selectable by identifying offspring growing at a temperature above 25°C following introduction in a pha-1 et123ts mutant *C. elegans* worm.

The present invention may be more clearly understood by the following examples which are purely exemplary with reference to the accompanying figures, wherein:

Figure 1 is a nucleotide sequence of plasmid PGN1 in accordance with the present invention.

Figure 2 is a nucleotide sequence of plasmid PGN100 in accordance with the present invention.

Figure 3 is a schematic representation of the vectors used and the transformation regime used in the methods according to the present invention.

Figure 4 is an illustration of the expression vector used in accordance with the invention.

Figure 5 is a schematic illustration of the T7 RNA polymerase expression vectors used for transforming *C. elegans*.

Figure 6 is an illustration of plasmid PGN1.

Figure 7 is a diagrammatic representation of an enhanced vector for dsRNA inhibition encoding sup-35 dsRNA.

Figure 8 is an illustration of a vector for integration into the genome of *C. elegans*.

EXAMPLE A: Construction of an ordered and hierarchical pooled cDNA library and applications thereof.

A random ordered and pooled library:

The vector is an *E. coli* vector harboring two T7 promoters, with a multiple cloning site (MCS) in between. The two promoters are orientated towards each other, and towards the MCS. In the presence of T7 RNA polymerase, expressed in *E. coli*, *C. elegans* or any other organism, RNA will be produced, starting from the two T7 promoters. As these are oriented in the opposite sense, both strands of RNA will be produced from the DNA inserted (cloned) into the MCS

in between the two promoters which results in the generation of double stranded RNA (dsRNA) upon binding of the T7 RNA polymerase thereto.

A *C. elegans* cDNA library is constructed in the MCS using standard molecular biological techniques. The library is transformed into *E. coli*, and the resulting *E. coli* are grown in culture and stored in 96 multi-well plates. At this stage, plasmid DNA can be isolated and stored in 96-multi-well plates corresponding to those of the *E. coli* colonies. Approximately 100,000 colonies are scored. In this way, the library will harbor approximately 5 times the total expressed cDNA variation of *C. elegans*, which gives the opportunity for low expressed sequences to be present in the library. This will result in approximately 1041 96-well plates. The plates are hierarchical pooled as necessary. For the present pooling of the clones is arranged in a range of 10 to 100. If the hierarchical pooling is per 8 or 12 (numbers are more convenient as 96-well plates have a 8 to 12 grind), this will result in approximately 87 multi-well plates and approximately 8352 wells. If hierarchical pooling is per 96 wells, which is a full plate, this results in approximately 11 plates and approximately 1041 wells. At any stage of the hierarchical pooling, plasmid DNA can be isolated, which would be less elaborate as less plates are used, but will result in a loss of complexity although this should not be the case in the pooling per 12. The pooling of the DNA can also be carried out with the original DNA.

The experiments below describe how the hierarchical pooling should be performed, both for the DNA and for the *E. coli* library.

An ordered library for RNAi technology, harboring every gene of the C. elegans genome, with applications thereof

As the genome-sequencing project is coming to an end, this information can be used in the T7 RNA inhibition technology. Every gene of the *C. elegans* genome can be cloned using PCR technology. In preference, exons will be cloned with a minimal length of 500bp. If the exons are too small, smaller fragments will be isolated with PCR, or even parts of introns and neighboring exons will be isolated with PCR technology so that at least a sufficient part of the translated region of the gene is cloned. For this, at least 17000 PCR reactions need to be performed. This collection of PCR products will be cloned in a T7 vector as described (two T7 promoters oriented towards each other with a multiple cloning site in between). Every PCR product will be cloned independently, or can be used to generate a random library, analogous to the described cDNA library. If every PCR product is cloned individually, the resulting bacteria and plasmid DNA can be pooled in various ways. First of all this collection of individual cloned PCR products in the T7 RNAi vector can be pooled randomly, as described in the random library, but this pooling can also be done in a more rational way. For instance, the genes of the *C. elegans* genome can be analyzed using bioinformatic tools (in silico biology). Various genes of the genome will belong to a gene family, or will have homologues in the genome. These members of the gene family will be pooled, or the members, being homologues will be pooled. In this way the total number of about 17000 clones is reduced to a more

useable quantity. This library can be used to screen for phenotypes in the methods according to the invention. The resulting phenotype gives a functional description to the gene or gene family or gene homologues of the *C. elegans* genome. As the library consists of a part of every gene in the genome, this method enables description of the full genome in functional-phenotypic terms. For this the double stranded RNA (dsRNA) needs to be introduced in the worm. This introduction of clones alone, or pooled clones, being random pooling or rational pooling can be achieved in several ways as described.

Example of a vector for the expression of double stranded RNAi

Any vector containing a T7 promoter may be used, and which contains a multiple cloning site (there are many commercially available). Primers containing the T7 promoter and a primer with the reverse complementary strand, both with the appropriate ends are designed. These primers can be hybridized, and if well designed, cloned in the vector of choice. The minimal sequence for a T7 promoter is TAATACGACTCACTATAGGGCGA. Although any vector can be used for the construction of a T7 expression vector there follows an example of how to achieve this with the vector pGEM-3Zf(-).

- Vector pGEM-3Zf(+) (PROMEGA) was digested with HindIII and SAlI
- Primers oGN1 and oGN2 were mixed together at a final concentration of 1 μg/30 μl boiled and cooled down slowly to room temperature.
- The primer was ligated into the vector using standard ligation procedures. The resulting vector is

pGN1 (shown in Figure 1) and contains two T7 promoters oriented towards each other, and harbors a multiple cloning site in between.

Sequences of oGN1 and oGN2 are:

- oGN1: AGC TGT AAT ACG ACT CAC TAT AGG GCG AGA AGC TT
- oGN2: TCG AAA GCT TCT CGC CCT ATA GTG AGT CGT ATT AC

Example of the construction of a library

RNA may be isolated from every organism that is sensitive to RNAi. In general the isolated RNA is then copied into double stranded cDNA, and subsequently prepared in suitable vectors for cloning. Several procedures exist and molecular biology kits can be purchased from various firms including promega, clontech, boehringer Mannheim, BRL, etc which enable:

- isolation of RNA,
- eventually polyA RNA can be isolated (several techniques and kits available)
- first strand synthesis with AMV reverse transcriptase, random hexameric primers and/or oligo(dT) primer
- second strand synthesis with Rnase H, DNA PolymeraseI,
- flush ends with T4 DNA Polymerase
- addition of an adaptor with T4 DNA ligase.
- eventually treatment with T4 polynucleotide Kinase
- cloning of the cDNA into the vector.

The resulting ligation mixture can be considered as the cDNA library. The ligation contains all cDNA of the procedure ligated into the vector of interest. To order the library, the ligation need to be transformed into *E. coli* strains.

Application of this E. coli or DNA library

- T7 RNA producing strain:
- standard strain is BL21(DE3): F-ompT[lon]hsdS(r-m-; and *E. coli* B strain) λ (DE3). Eventually variants of BL21 (DE3) can be used, although BL21 (DE3)pLysS is used.
- any other *E. coli* strain which produces the T7 RNA polymerase, which may be available needs to be constructed. This can be generated rather easily, using a phage, which is commercially available, in this case we will use the λCE6 (provided by Promega). Almost every *E. coli* strain can be transfected with this phage and will produce T7 RNA polymerase.
- a RNAseIII mutant *E. coli*:
- Various strains are in principle available, we chose in a first experiment to use strain AB301-105: rna-19, suc-11, bio-3, gdhA2, his95, rnc-105, relA1, spoT1, metB1. (Kinder *et al.* 1973 Mol. Gen. Genet 126:53), but other strains may suit better. This strain will be infected with λCE6 and so a T7 producing variant will be constructed. In a further process we will look at the possibilities for other strains, if this seems necessary.
- Wild type *C. elegans* worms can be grown on the bacteria pools. The bacteria is expressing the T7 RNA polymerase. This results in large quantities of dsRNA in the gut of the *C. elegans*, which will diffuse in the organism and results in the inhibition of expression (as reported previously). This library can now be used for the screening of several phenotypes. This is a much faster technique to detect relevant genes in certain pathways, than the known *C. elegans* technology. Moreover, if an interesting phenotype is found, the responsible gene can be cloned easily.

Using the hierarchical pooling one can easily find in a second screen the relevant clone of the pool. The inserted DNA of this clone can be sequenced. This experiment results in genetic and biochemical DATA in one step.

- Wild type *C. elegans* strain can be combined with compounds, screening for phenotype, drug resistance and or drug sensibility,
- The *C. elegans* strain can be a mutant strain, screening for an enhanced phenotype, reduced phenotype, or a new phenotype.
- The *C. elegans* strain can be a mutant strain, and the library screen can be combined with compounds. So one can screen for drug resistance, drug sensibility, enhanced phenotype, reduced phenotype, or a new phenotype.
- The *E. coli* strain may be any T7 RNA polymerase expressing strain, like BL21 (DE3) but the formation of double strand RNA may be enhanced by using a special *E. coli* strain that is RNAseIII negative. RNAseIII recognizes specific loops in dsRNA. Eventually, an *E. coli* strain can be used that is deleted in other RNAses than RNAseIII or an *E. coli* can be used that is deleted in one or more RNAses.
- The expression of the T7 RNA polymerase in most known *E. coli* strains and constructs that are available to generate T7 RNA polymerase producing *E. coli* strains mostly comprise an inducible promoter. This way the production of the T7 RNA polymerase is regulated, and thus the production of the dsRNA. Advantageously, this feature can be used to "pulse" feed the *C. elegans* worms at specific stages of growth. The worms are grown on the non-induced *E. coli* strains. When the worm has reached the stage of interest, the T7 RNA production in the bacteria is

induced. This allows the studying of the function of any gene at any point in the life cycle of the animal.

Screening the library for homologues of putative interesting human genes, and assign function to these genes

Hundreds of genes have been isolated in various projects, being genomic projects, differential expressed arrays, hybridization studies, etc. The described cDNA library can provide a way to validate and or assign function to these genes in a fast and efficient manner. First of all the worm homologue or homologues or the genes need to be identified by bioinformatic tools (in silico biology). PCR primers are developed and the cDNA fragment is isolated using PCR technology. PCR can be performed on the hierarchical pools. The positive pool or individual wells harboring the bacteria that has the appropriate cDNA is fed to *C. elegans* and the phenotype is scored.

PCR can be performed on cDNA isolated from *C. elegans*. The resulting DNA can be cloned in the T7 vector and transformed in the dsRNA producing *E. coli*, on which the *C. elegans* worms are then fed. Depending on which way is faster and more reliable a choice needs to be made.

If the gene belongs to a gene family, the worm may need to be fed on a mixture of bacteria. Each of them harboring a part of the member of the gene family.

E. coli strains, growth conditions, combinations with compounds can be performed as described above.

If the library rational is used, in which all the genes of *C. elegans* are cloned in a organized and structured way, the *C. elegans* homologue and

eventually the other homologues, orthologues, and members of the gene family can be traced back easily in the library using in silico biology. No PCR is involved in this step, and the bacteria and or DNA can be isolated on which the worm will be grown.

Examples

The idea of the series of experiments is to test both the RNAi vector, the various *E. coli* strains that have been constructed.

1) Construction of a test plasmid.

Any cDNA that gives a clear phenotype in the worm when knocked-out, or used in a RNAi experiment can be used. It is known that unc-22 is a good candidate, but a lot of other genes are possible. We chose for a sensitive system that can be used in a later stage. We tested the system with sup-35 in a pha-1 background. Exon 5 of the sup-35 was isolated by PCR and cloned in the T7 promoter vector pGN1. The resulting vector was designated pGN2. pha-1 (e2123) mutant worms cannot produce offspring at temperatures higher than 25°C. This is due to a developmental problem in embryogenesis. When sup-35 is knocked-out, or inhibited in this strain, offspring may grow at this temperature. Combination of pha-1 mutant worms and sup-35 RNAi is a good system to validate the various options.

2) Testing the RNAi using an *E. coli* strain that produces dsRNA.

- pGN2 was introduced in *E. coli* strain BL21(DE3) and T7 RNA polymerase was induced with IPTG. *C. elegans* worms (pha-1 (e2123)) were inoculated on this

bacteria, and grown at the restricted temperature of 25°C. As this mutant is an embryonic mutant at this temperature, no offspring will be observed. If the sup-35 gene is efficiently inhibited by the dsRNA present in the *E. coli*, offspring will be observed.

- pGN2 was introduced in *E. coli* strain AB301-105(DE3) and T7 RNA polymerase was induced with IPTG. *C. elegans* worms (pha-1 (e2123)) were inoculated on this bacteria, and grown at the restricted temperature of 25°C. As this mutant is an embryonic mutant at this temperature, no offspring will be observed. If the sup-35 gene is efficiently inhibited by the dsRNA present in the *E. coli*, offspring will be observed.

3) Improving the worm strain for better uptake of dsRNA.

Before plating the pha-1 *C. elegans* on the *E. Coli* strain that produce the double stranded sup-35 RNA. The worm was mutagenised with EMS (Methane Sulfonic Acid Ethyl). The offspring of this mutagenised worm is than plated on the bacteria. The worm that feed on this bacteria that gives a larger offspring has a mutation that may be the improvement of dsRNA uptake, and can be used for further experiments.

Stable integration of the dsRNA producing vector into the genome of the T7 RNA polymerase producing worm

An *E. coli* vector can be constructed harboring the following features; Two T7 promoters directed towards each other, with a restriction site or a multiple cloning site in between. Furthermore, the vector may contain the *C. elegans sup35* genomic DNA,

engineered in such a way that it contains several stopcodons at various intervals, so that no full length protein can be expressed from the *sup35* genomic DNA fragment as illustrated in Figure 8. Any cDNA or cDNA fragment can be cloned in the multiple cloning site between the two T7 promoters. When this vector is introduced in a *C. elegans* strain which expresses T7 RNA polymerase, the cDNA or DNA fragment cloned between the two T7 promoters will be transcribed, generating dsRNA from the cloned fragment.

The vector is designed to be used in pha-1 (e2123) mutant worms expressing T7 RNA polymerase. The expression of the T7 RNA polymerase may be constitutive or regulated, general or tissue specific. These pha-1 (e2123) worms cannot produce offspring at temperatures higher than 25°C, which is due to a development problem in embryogenesis. When sup-35 is inhibited or knocked-out in this stain, offspring may grow at this temperature.

When the vector is introduced in the worm, the vector may integrate by homologous recombination (Campbell-like integration). It has been shown that homologous recombination occurs in *C. elegans*, although at low frequencies (Plasterk and Groenen, EMBO J. 11:287-290, 1992). Homologous recombination at the *sup35* gene will result in a knock-out of the gene as the two resulting *sup-35* genes will harbor the stopcodons. The resulting worm, and its offspring, if this recombination happens in the eggs, will have a copy of the vector integrated in the genome. This can be selected as only the worms for which the sup-35 has been knocked-out will have offspring at temperatures higher than 25°C. Furthermore, the resulting worm will stably produce double stranded RNA from the DNA fragment cloned between the two T7 promoters. This

worm can now be considered as a stable transgenic worm strain with a reduction of function of the gene, from which a fragment has been cloned between the two T7 promoters.

The DNA may be delivered to the worm by several techniques, including injection, ballistic transformation, soaking in the DNA solution, feeding with bacteria. New and other methods that increase the transformation efficiencies can be considered.

The target *C. elegans* strain may in addition, have other mutations than the pha-1 (e2123) mutation, and may express other genes than T7 RNA polymerase.

EXAMPLE B: a Yeast two-hybrid-RNAi vector, with applications

A yeast two hybrid vector can be constructed harboring the two T7 promoters. The vectors can be designed to replicate both in yeast and in *E. coli*. In general cDNA libraries for the yeast two hybrid system are made in the Gal4 or LexA vectors. The library is constructed in vectors having the activation domain of one of these genes. A vector can easily be constructed that can still perform in the yeast two hybrid screen but which also contains the two T7 promoters orientated to each other, with a cloning site in between. The structure of the plasmid will then be "backbone, (GAL4-T7), MCS, T7, backbone". A *C. elegans* cDNA library constructed in this vector can be used as a standard yeast two hybrid library in an experiment to isolate interacting proteins with a given protein. Once a clone is isolated, the plasmid can be introduced in an *E. coli* strain expressing the T7 RNA polymerase, and hence will produce dsRNA of the cloned fragment. The bacteria producing this dsRNA

can be fed to the worm and phenotypes can be scored. As in Example A), this validation procedure for a newly isolated yeast two hybrid clone is remarkably shorter than the standard procedure, which requires PCR and or cloning steps, RNA experiments and or knock-out experiments. In most cases isolated clones are sequenced first, and on the basis of the sequence, a decision is made to continue with further experiments. In the present invention every isolated clone can easily be introduced into the appropriate *E. coli* and fed to the worm. Validation is then done by phenotype analysis.

Examples of procedure

A yeast two hybrid was performed using a known gene as bait and the newly constructed library as the target. Proteins coded by the clones in the target that interact with the bait protein, will result in positive yeast clones expressing the reporter molecule such as that can be observed by LacZ staining with X-gal. The plasmid coding for the target protein can be isolated directly from the yeast strain and introduced in *E. coli*. The *E. coli* may be a T7 RNA polymerase producing *E. coli*. In this case, double stranded RNA will be produced from the DNA cloned in the multiple cloning site of the vector. When this dsRNA is fed to the worm using the methods described previously, the gene will be inhibited in the worm, resulting in a particular phenotype.

- This yeast two hybrid vector can be used to construct an ordered and hierarchically pooled library as described in Example A).
- A yeast strain could be constructed that conditionally produces T7 RNA polymerase. After yeast

two hybrid experiments, the expression of the T7 polymerase could be induced, resulting in the production of dsRNA in the yeast cell. Consequently the yeast could be fed to the worm. Some evidence is known that worm can feed on yeast, nothing is known about the stability of dsRNA in yeast.

Example C: Construction of a T7 RNA polymerase producing strain, and applications thereof

A *C. elegans* strain can be constructed that expresses T7 RNA polymerase. The expression can be general and constitutive, but could also be regulated under a tissue specific promoter, an inducible promoter, or a temporal promoter or a promoter that harbors one of these characteristics or combination of characteristics. DNA can be introduced in this *C. elegans* strain. This can be done either by injection, by shooting with particles, by electroporation or as aforementioned by feeding. If the DNA is a plasmid as described in Examples A) and B), i.e. a plasmid harboring a cloned cDNA fragment or a PCR fragment between two flanking T7 promoters, then dsRNA of this cDNA or PCR fragment is formed in the cell or whole organism resulting in down regulation of corresponding gene. The introduced DNA can have an efficient transient down regulation. The introduced DNA can form an extrachromosomal array, which array might result in a more catalytic knock-out or reduction of function phenotype. The plasmid might also integrate into the genome of the organism, resulting in the same catalytic knock-out or reduction of function phenotype, but which is stably transmittable.

- Plasmid DNA harboring a cDNA or a part of a cDNA or an EST or an PCR fragment of *C. elegans* cloned between

two T7 promoters as described in Examples A) and B) can be introduced in the T7 RNA polymerase worm, by standard techniques. Phenotypes can be analysed -DNA from an ordered and pooled library as in Example A) can be introduced in the T7 RNA polymerase worm, by standard techniques (injection, shooting). Phenotypes can be analysed. With the hierarchical pool, the original clone can be found easily.

- The same procedure can be performed with a mutant worm expressing the T7 RNA polymerase. Screening for enhanced, reduced or new phenotypes.
- The procedure can be used to enable screening of compounds. Screening with either a wild-type strain or a mutant strain for enhanced or new phenotypes.
- The DNA could be introduced in the worm by new methods. One of which is the delivery of DNA by *E. coli*. In this case the hierarchical pooled library is fed to the animal. To prevent digestion of the *E. coli* DNA in the gut of the nematode, preferentially a DNAse deficient *C. elegans* will be used, such as nuc-1 (e1392). This procedure would be one of the most interesting as it would be independent of transformation efficiencies of other techniques, and generally faster and less labourious.

2) Putative enhancements of the method.

- A vector is designed, so that it harbors the sup-35 cDNA or a part of this cDNA, cloned in between two T7 promoters. The rest of the vector is as described in Examples A) and B). This vector can be introduced into a pha-1ts mutant *C. elegans*. A temperature selection system exists in this case and only those worms which have taken up the DNA and express the double stranded sup-35 RNA will survive at restricted temperatures. The hierarchical pooled library can be delivered by

any method described above.
- The vector can be used to construct a library that is introduced in a T7 RNA polymerase expressing *E. coli*. In this case we have an analogous screening as in part A)with an additional screening for worms where the dsRNA of sup-35 is active.
- The DNA and or dsRNA of sup-35 could be delivered on a different plasmid. For the feeding; both DNA feeding (Example C) or dsRNA feeding Example A) and B), this means that the two plasmids could be present in one bacterium, or that the worm is fed on a mixture of bacteria, one of which harbors the sup-35 construct.

Example of the construction of a T7 RNA producing C. elegans

To produce T7 RNA polymerase in the worm, several possibilities are possible. The T7 polymerase can be expressed under various promoters, being inducible promoters, constitutive promoters, general promoters and tissue (cell) specific promoters, or combinations of those. Examples of these promoters are the heatshock promoter hsp-16, the gut promoter ges 1, the promoter from cet858, but also the promoter of dpy7 and the promoter element GATA1. In this example the T7 RNA polymerase is expressed under the control of the hsp-16 promoter that is available in the pPD49.78 vector. The T7 RNA polymerase is isolated as a PCR product using the primers of GN3 an GN4.

The resulting PCR product is digested with NheI and NcoI, as is the vector in which we want to clone, being the Fire vector pPD49.78. The resulting vector is pGN100 illustrated in Figure 2 oGN3: CAT GGC AGG ATG AAC ACG ATT AAC ATC GC oGN4: ATG GCC CCA TGG TTA CGG GAA CGC GAA GTC CG pGN100 is included.

The vector is introduced into the worm using standard techniques, like micro injection.

The following strains were then constructed:

-Wild-type (pGN100)
-nuc-1 (e1392)(pGN100)
-pha-1 (e2123)(pGN100)
-pha-1; nuc-1 (pGN100)

All of these strains are able to produce T7 RNA polymerase when temperature induced or alternatively by metals such as application of heavy cadmium or mercury. The procedure for temperature induction is to shift the animal to 30-33°C for at least one hour, then the animal can be shifted back to standard temperatures (15-25°C).

The wild type strain producing T7 RNA polymerase can be used for the production of any RNA in the worm. More specifically, the plasmids from the described libraries can be introduced in these worms, and phenotypes can be scored.

The nuc-1 mutant worm will be used to introduce DNA via bacteria on which the worm feed. As the nuc-1 worm does not digest the DNA, the plasmid DNA can cross the gut wall. If taken up by the cells that produce the T7 RNA polymerase, dsRNA will be produced thus inhibiting the gene from which the RNA was transcribed.

The pha-1 mutant strain that produced T7 RNA polymerase can be used to enhance the procedures as described above, DNA can be introduced by shooting, micro injection or feeding. More specifically this strain can be used for the vectors that produce dsRNA from sup-35 and from the gene of interest, the later

can be a PCR product, a cDNA, or a library as described.

The pha-1; nuc-1 mutant producing T7 RNA polymerase can be used for the bacterial delivery of the DNA. DNA will preferentially be the plasmid that produce dsRNA from both sup-35 and the gene of interest. The worm strain will preferentially produce the T7 RNA polymerase in the gut. Delivery will preferentially happen by feeding the worm on bacteria harboring the plasmid.

Application of the RNAi technology in plants

Nematodes are responsible a large part of the damage inflicted on plants and more particularly to plants used in the agricultural industry. The RNAi procedures according to the invention can be applied to plants to prevent these parasitic nematodes from feeding longer. In a first step, a DNA fragment is isolated from the parasitic plant nematode that is critical for the animals survival or growth, or to feed or to proliferate. Any gene from which the expression is essential is suitable for this purpose.

A part of this gene, an exon or cDNA is cloned. This DNA fragment can be cloned under the influence of a tissue specific promoter preferably a root specific promoter even more preferably between two root specific promoters. The DNA of the cloned gene under the control of the root specific promoter can be introduced in the plant of interest, using plant transgenics technology. For every parasitic nematode, a different piece of DNA may be required and likewise for every plant race, a different promoter will be needed.

The root will produce RNA or dsRNA from the

introduced piece of DNA when root specific promoter is utilised. As the nematode feeds on the plant, the RNA and/or dsRNA will be consumed or ingested by the nematode. The RNA and/or dsRNA can enter the cells of the nematode and perform its inhibitory action on the target DNA. Depending on the nature of the cloned DNA piece of worm, the nematode will not be able to survive, to eat, proliferate, etc in any case preventing the animal of feeding longer on the plant, and thus protecting the plant.

Claims

1. A method of identifying DNA responsible for conferring a particular phenotype in a cell which method comprises

a) constructing a cDNA or genomic library of the DNA of said cell in a suitable vector between two promoters capable of promoting transcription of said cDNA or DNA to double stranded (ds) RNA upon binding of an appropriate transcription factor to said promoters,

b) introducing said library into one or more of said cells comprising said transcription factor, and

c) identifying and isolating a particular phenotype of said cell comprising said library and identifying the DNA or cDNA fragment from said library responsible for conferring said phenotype.

2. A method according to claim 1 wherein said library is organised into hierarchical pools prior to step b).

3. A method of assigning function to a known DNA sequence which method comprises

a) identifying a homologue(s) of said DNA sequence in a cell,

b) isolating the relevant DNA homologue(s) or a fragment thereof from said cell,

c) cloning said homologue or fragment into an appropriate vector between two promoters capable of promoting transcription of dsRNA upon binding of an appropriate transcription factor to said promoters,

d) introducing said vector into said cell from step a) comprising said transcription factor, and

e) identifying the phenotype of said cell

compared to wild type.

4. A method according to any of claims 1 to 3 wherein said cell is contained in an organism.

5. A method according to claim 4 wherein said organism is adapted to express said transcription factor.

6. A method according to claim 4 or 5 wherein said organism comprises a plant, animal, fungus, bacteria or yeast and preferably the nematode worm *C. elegans*.

7. A method according to claim 6 wherein said organism is any of a wild-type, a nuc-1; phal-ts; mutant of *C. elegans* or a combination thereof.

8. A method according to any of claims 1 to 3 wherein said DNA library or said DNA homologue or DNA fragment is transformed in a microorganism for introduction into an organism.

9. A method according to claim 8 wherein said microorganism or said organism is adapted to express said transcription factor.

10. A method according to claim 9 wherein said microorganism is *E. coli*.

11. A method according to any of claims 1 to 10 wherein said library or DNA homologue or DNA fragment is constructed in a suitable DNA vector which comprises a sequence of nucleotides which encode said transcription factor.

12. A method according to any of claims 1 to 10 wherein said transcription factor is encoded by a further vector independent of the DNA vector including said library or which cell or organism has been mutated to express said transcription factor.

13. A method according to any preceding claim wherein said vector comprises a selectable marker.

14. A method according to claim 13 wherein said selectable marker comprises a nucleotide sequence capable of inhibiting or preventing expression of a gene in said cell or organism and which gene is responsible for conferring a known phenotype.

15. A method according to claim 14 wherein said nucleotide sequence comprises a sequence which is a part of or identical to said gene conferring said phenotype, and which nucleotide sequence is itself located between two identical promoters capable of initiating transcription of double stranded RNA upon binding of an appropriate transcription factor to said promoters.

16. A method according to claim 14 wherein said nucleotide sequence is a part of or identical to said gene sequence conferring said phenotype, and which nucleotide sequence is such that following integration of said vector by homologous recombination in the chromosome of said cell or organism said nucleotide sequence inhibits expression of said gene sequence conferring said phenotype.

17. A method according to claim 16 wherein said nucleotide sequence comprises stop codons sufficient

to prevent translation of said nucleotide sequence following integration into said chromosome.

18. A method according to any of claims 14 to 17 wherein said known gene sequence comprises a sup-35 gene or a fragment thereof which is selectable by identifying offspring growing at a temperature above 25°C following introduction in a pha-1 et123ts mutant *C. elegans* worm.

19. A method according to any of claims 14 to 18 wherein said promoters are T7 promoters.

20. A method according to any of claims 1 to 19 wherein said cell or organism is contacted with a specified compound for screening for a desired phenotype, such as resistance or sensitivity to said compound when compared to the wild type cell or organism.

21. A method according to any preceding claim wherein said transcription factor is inducible.

22. A method according to any preceding claim wherein said transcription factor is a phage derived RNA polymerase, such as T7 polymerase.

23. A method according to claim 10 wherein said *E. coli* strain is an RNAase III and preferably an RNAase negative strain.

24. A method of validating clones identified in yeast two hybrid vector experiments which method comprises

a) providing a construct including the DNA

encoding the protein identified in the two hybrid vector experiment, which construct is such that said DNA is provided between two promoters capable of promoting transcription of said DNA to double stranded RNA upon binding of an appropriate transcription factor to said promoters,

b) transforming a cell such as a bacterial cell, or an organism, comprising said transcription factor with said construct, and

c) identifying a phenotypic change in said cell or organism when compared to a wild type.

25. A method according to claim 24 wherein said transcription factor is inducible in said cell or organism.

26. A method according to claim 24 or 25 wherein said promoter is a phage polymerase promoter and said transcription factor is a RNA polymerase, and preferably T7 polymerase.

27. A method according to any of claims 24 to 26 wherein said cell is an *E. coli* cell.

28. A method according to any of claims 24 to 26 wherein said organism is *C. elegans*.

29. Plasmid pGN1 as illustrated in Figure 1.

30. Plasmid pGN100 as illustrated in Figure 2.

31. The yeast two hybrid vector plasmid illustrated in Figure 4.

32. A plasmid as illustrated in Figure 7.

33. A plasmid as illustrated in Figure 8.

34. A DNA or RNA molecule identifiable or obtainable according to the methods of any of claims 1 to 28.

35. A DNA or RNA molecule according to claim 34 for use as a medicament.

36. A cell or organism identifiable as exhibiting a desired phenotype according to the method of claim 20.

37. A method of alleviating infestation of plant pests, which method comprises

a) identifying a DNA sequence from said pest which is critical for its survival, growth, proliferation or reproduction,

b) cloning said sequence from step a) or a fragment thereof in a suitable vector between two promoters capable of transcribing said sequence to RNA or dsRNA upon binding of an appropriate transcription factor to said promoters, and

c) introducing said vector into the plant.

38. A method according to claim 37 wherein said pest is a nematode worm.

39. A method according to claim 38 wherein said nematode comprises any of Tylenchulus ssp. Radopholus ssp., Rhadinaphelenchus ssp., Heterodera ssp., Rotylenchulus ssp., Pratylenchus ssp., Belonolaimus ssp., Canjanus ssp., Meloidogyne ssp., Globodera ssp., Nacobbus ssp., Ditylenchus ssp., Aphelenchoides ssp., Hirschmenniella ssp., Anguina ssp., Hoplolaimus ssp.,

Heliotylenchus ssp., Criconemellassp., Xiphinemassp., Longidorus ssp., Trichodorus ssp., Paratrichodorus ssp., Aphelenchs ssp.

40. A method according to claim 37 wherein said DNA sequence or fragment thereof from step b) is cloned between two tissue, preferably root specific promoters.

41. An expression vector comprising two identical promoters oriented such that they are capable of initiating transcription of a DNA sequence located between said promoters to double stranded RNA upon binding of an appropriate transcription factor to said promoters.

42. An expression vector according to claim 41 which comprises a nucleotide sequence encoding a selectable marker.

43. An expression vector according to claim 42 wherein said nucleotide sequence is located between two identical promoters oriented such that they are capable of initiating transcription of DNA located between said promoters to double stranded RNA upon binding of an appropriate transcription factor to said promoters.

44. An expression vector according to claim 42 or 43 wherein said selectable marker comprises a nucleotide sequence encoding sup-35, for introduction into *C. elegans* having a pha-1 mutation.

45. An expression vector for expressing an appropriate transcription factor for use in a method

according to any of claims 1 to 26 and 37 to 40 which vector comprises a sequence of nucleotides encoding said transcription factor operably linked to suitable expression control sequences.

46. An expression vector according to claim 45 wherein said expression control sequences include promoters which are inducible, constitutive, general or tissue specific promoters, or combinations thereof.

47. An expression vector according to any of claims 41 to 46 wherein said transcription factor comprises a phage polymerase, and preferably T7 RNA polymerase.

48. An organism or cell transformed or transfected with a plasmid according to any of claims 29 to 33 or an expression vector according to any of claims 41 to 47.

49. An organism according to claim 48, wherein said organism is *C. elegans*.

50. A method of introducing dsRNA or DNA capable of producing dsRNA into an organism which method comprises feeding said organism with a suitable microorganism comprising an expression vector according to any of claims 41 to 44 or feeding said organism with an expression vector according to any of claims 41 to 44.

51. A method according to claim 50 wherein said microorganism or said organism is adapted to express said transcription factor.

52. A method according to claim 51 wherein either said microorganism or said organism comprises an expression vector according to any of claims 45 to 47.

53. A method according to any of claims 50 to 52 wherein said organism is *C. elegans* and said microorganism is *E. coli*.

54. A method according to claim 53 wherein said *E. coli* strain is an RNAseIII negative strain.

55. A method according to any of claims 50 to 52 wherein said organism is a *C. elegans* nuc-1 mutant when said DNA is fed directly thereto.

56. A selection system for identifying transformation of a cell or organism with a vector according to claim 41 or 42 which system comprises a vector according to claim 41 or 42 wherein said selectable marker comprises a nucleotide sequence capable of inhibiting or preventing expression of a gene in said cell or organism which gene is responsible for conferring a known phenotype.

57. A selection system according to claim 56 wherein said nucleotide sequence comprises a sequence which is a part of or identical to said gene conferring said known phenotype, and which nucleotide sequence is itself located between two identical promoters capable of initiating transcription of double stranded RNA upon binding of an appropriate transcription factor to said promoters.

58. A selection system according to claim 56

wherein said nucleotide sequence is a part of or identical to said gene sequence which confers a known phenotype on said cell or organism, and which is nucleotide sequence such that following integration of said vector by homologous recombination in the chromosome of said cell or organism said sequence inhibits expression of said gene sequence conferring said known phenotype.

59. A selection system according to claim 58 wherein said nucleotide sequence comprises stop codons sufficient to prevent translation of said nucleotide sequence following integration into said chromosome.

60. A selection system according to claim 56 wherein said known gene sequence comprises a sup-35 gene or a fragment thereof which is selectable by identifying offspring growing at a temperature above 25°C following introduction in a pha-1 et123ts mutant *C. elegans* worm.

61. A method according to claim 51 wherein said transcription factor is T7 RNA polymerase.

pGN1

gagtgcaccatatgcggtgtgaaataccgcacagatgcgtaaggagaaaataccgcatcaggcgaaattgtaaacgttaatattt
tgttaaaattcgcgttaaatatttgttaaatcagctcatttttaaccaataggccgaaatcggcaaaatcccttataaatcaaaagaat
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ccagtgaattgtaatacgactcactatagggcgaattcgagctcggtacccggggatcctctagagtcgaaagcttctcgccctat
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Figure 1

caccggctccagatttatcagcaataaaccagccagccggaagggccgagcgcagaagtggtcctgcaactttatccgcctcc
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Figure 1

PGN100
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Figure 2

ctgctgaggtcaaagataagaagactggagagattcttcgcaagcgttgcgctgtgcattgggtaactcctgatggtttccctgtgt
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Figure 2

ctagaaggtcctagatgcattcgtttgaaaatactcccggtgggtgcaaagagacgcagacggaaaatgtatctgggtctctttatt
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Figure 2

VECTOR:
(only important features)

BACTERIAL STRAIN
(only important phenotypes)

C. ELEGANS STRAIN

Simple form:

T7 — MCS — T7

E. coli (T7 RNA polymerase)

E. coli (T7 RNA polymerase, RNAseIII-)

wild-type

T7 RNA polymerase
nuc-1 mutant

E. coli (no special feature)

YTH form:

Gal4-T7 — MCS — T7

DNA

T7 RNA polymerase

Sup-35 form:

T7 — sup-35 — T7

T7 — MCS — T7

E. coli (T7 RNA polymerase)

E. coli (T7 RNA polymerase, RNAseIII-)

wild-type
pha1-ts mutant

E. coli (no special feature)

T7 RNA polymerase
pha1-ts mutant
nuc-1 mutant

DNA

T7 RNA polymerase
pha1-ts mutant

Figure 3

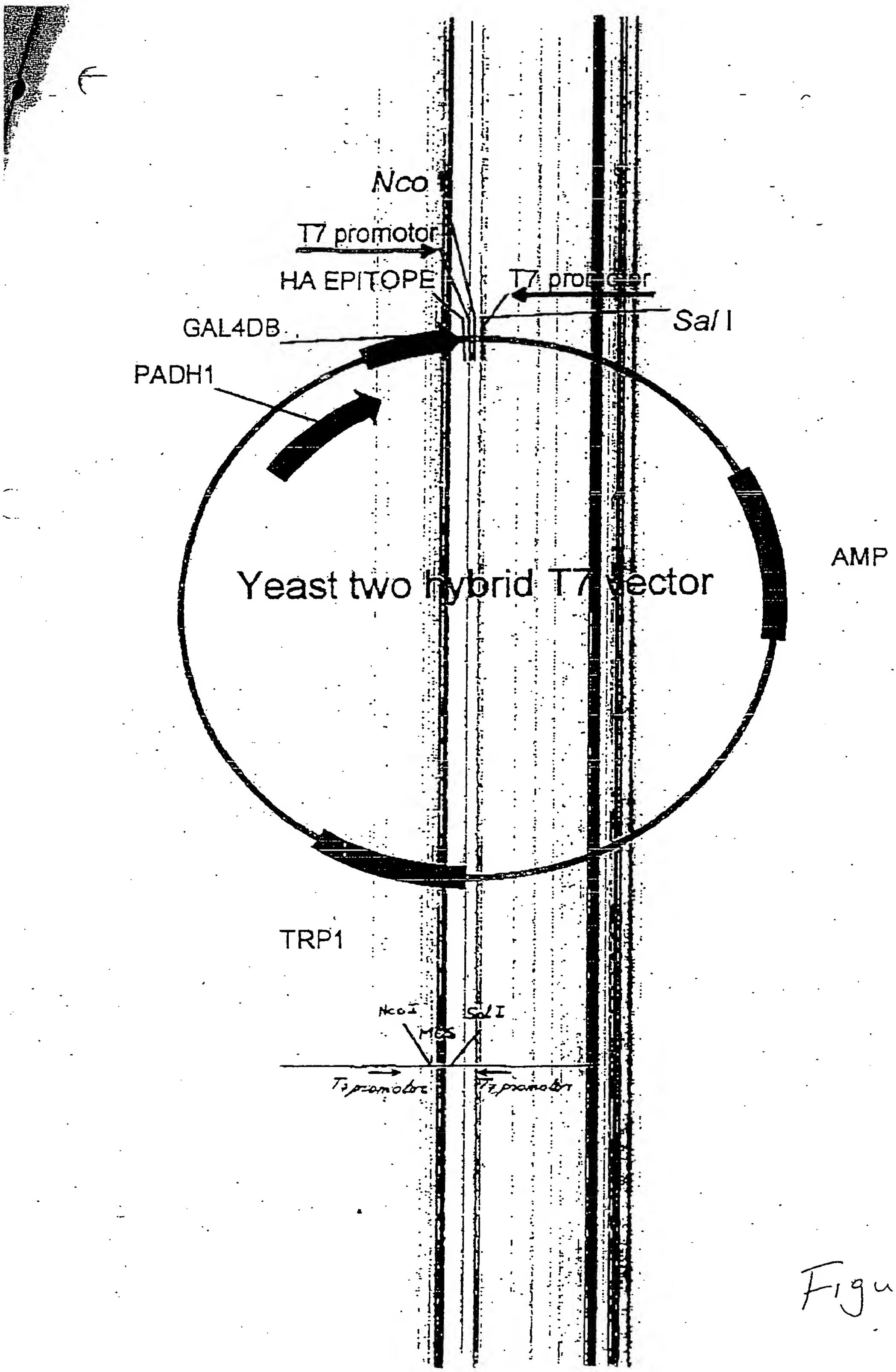

Nco
T7 promotor
HA EPITOPE
T7 prom Gter
Sal I
GAL4DB
PADH1
Yeast two hybrid T7 vector
AMP
TRP1
NcoI
SalI
MCS
T7 promoter
T7 promoter

Figure 4

General description of the C.elegans T7 RNA polymerase expression vector with 4 examples

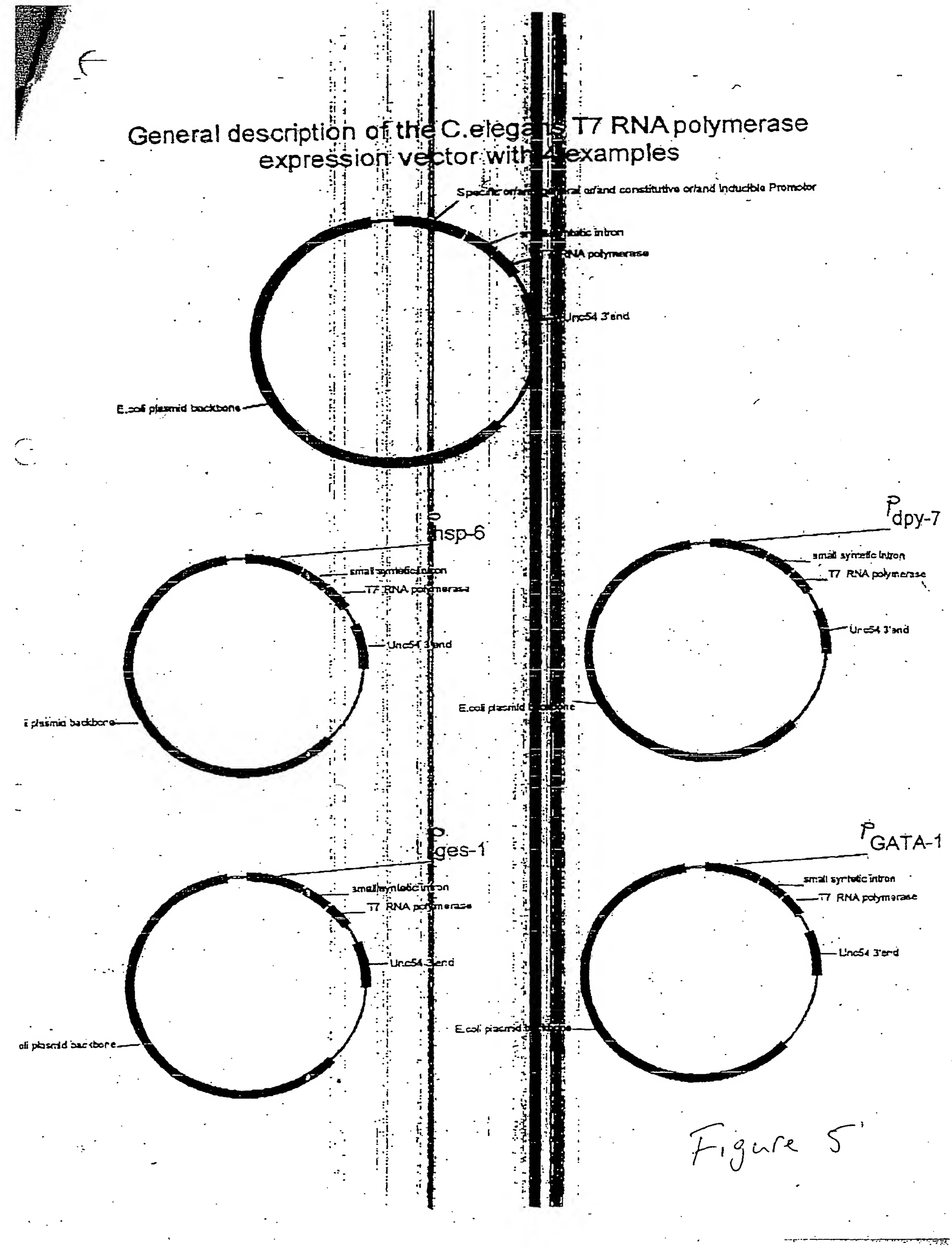


Figure 5

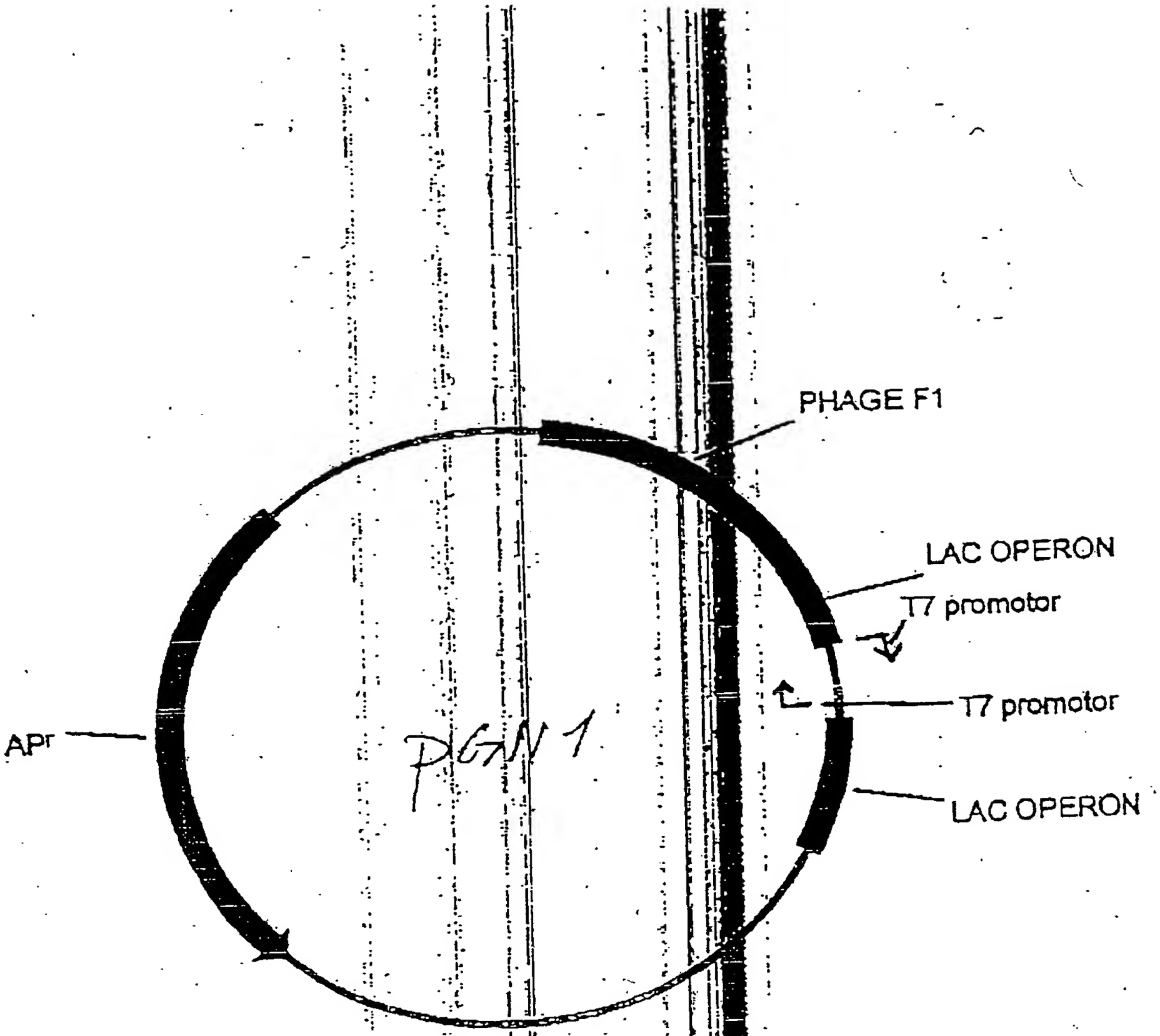
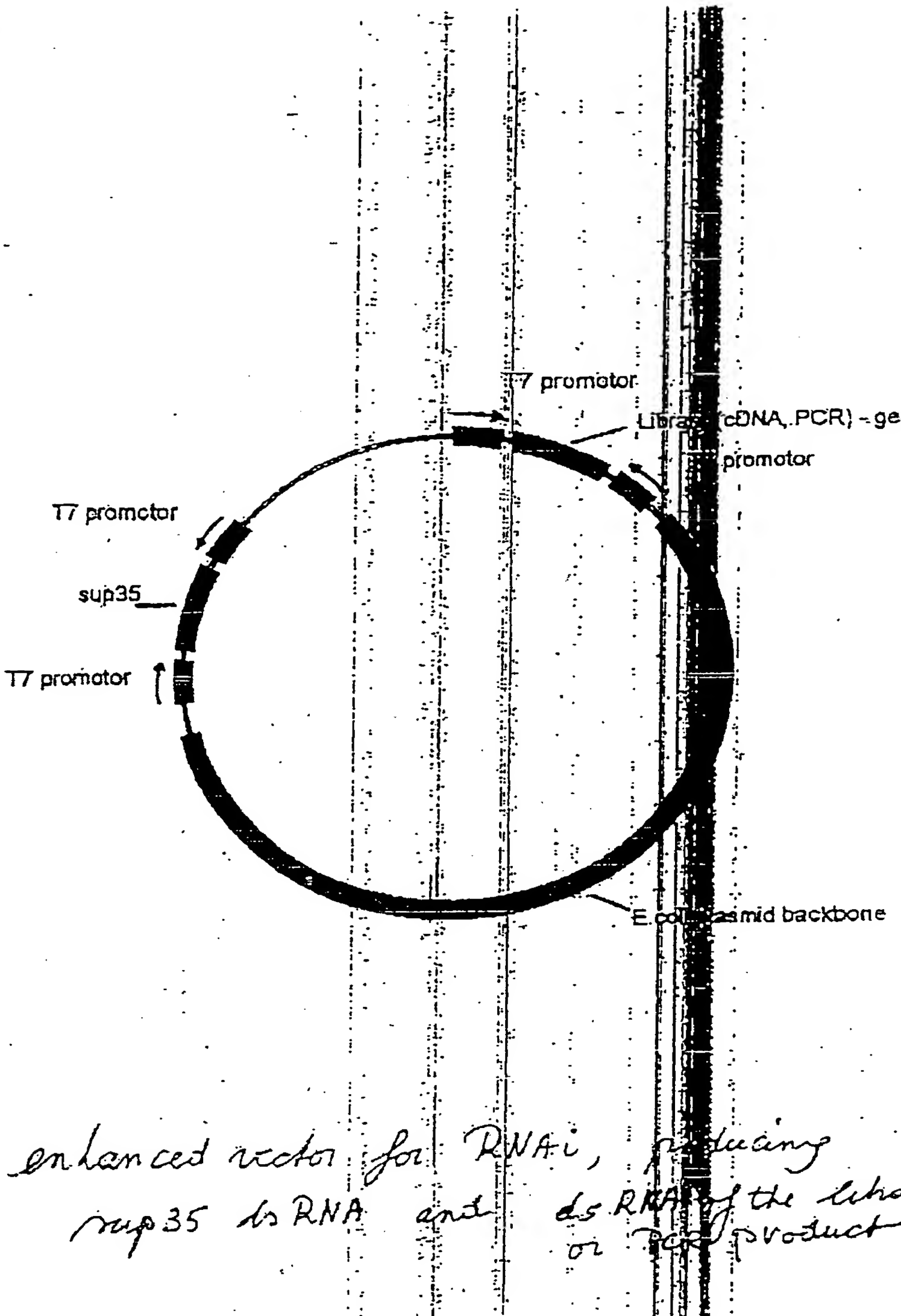

PHAGE F1
LAC OPERON
T7 promotor
T7 promotor
LAC OPERON
APr
PGN1

Figure 6



enhanced vector for RNAi, producing sup35 ds RNA and ds RNA of the library, gene of interest or PCR product

Figure 7

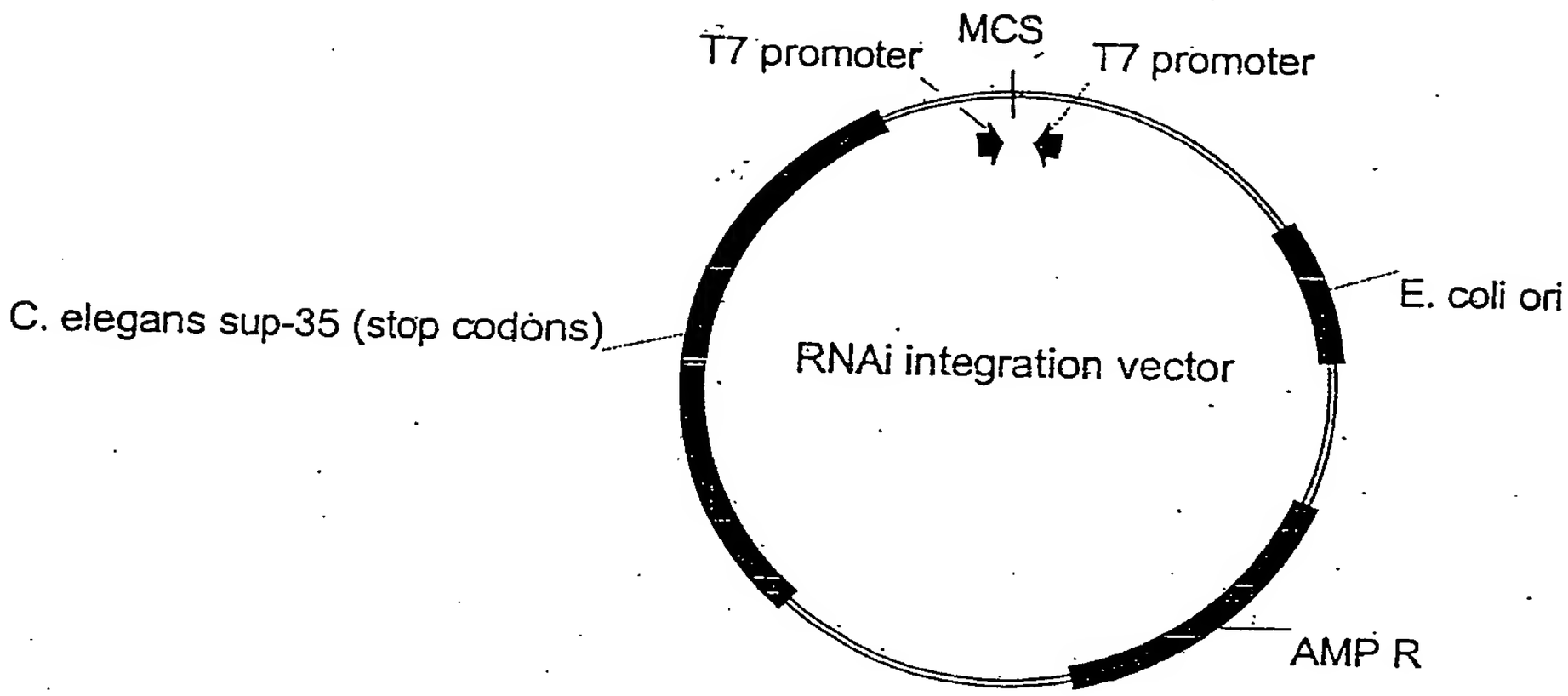

T7 promoter
MCS
T7 promoter
E. coli ori
C. elegans sup-35 (stop codons)
RNAi integration vector
AMP R

Figure 8